Mol. Cells *27*, 105-111
DOI/10.1007/s10059-009-0011-5

Molecules and Cells

Age-Dependent Pathogenesis of Murine Gammaherpesvirus 68 Infection of the Central Nervous System

Hye-Jeong Cho[1,5], Sungbum Kim[1,2,5], Sung-Eun Kwak[3], Tae-Cheon Kang[3], Hee-Sung Kim[2], Hyung-Joo Kwon[2], Yoon-Won Kim[2], Yong-Sun Kim[2], Eun-Kyung Choi[4], and Moon Jung Song[1,*]

Gammaherpesvirus infection of the central nervous system (CNS) has been linked to various neurological diseases, including meningitis, encephalitis, and multiple sclerosis. However, little is known about the interactions between the virus and the CNS *in vitro* or *in vivo*. Murine gammaherpesvirus 68 (MHV-68 or γHV-68) is genetically related and biologically similar to human gammaherpesviruses, thereby providing a tractable animal model system in which to study both viral pathogenesis and replication. In the present study, we show the successful infection of cultured neuronal cells, microglia, and astrocytes with MHV-68 to various extents. Upon intracerebroventricular injection of a recombinant virus (MHV-68/LacZ) into 4-5-week-old and 9-10-week-old mice, the 4-5-week-old mice displayed high mortality within 5-7 days, while the majority of the 9-10-week-old mice survived until the end of the experimental period. Until a peak at 3-4 days post-infection, viral DNA replication and gene expression were similar in the brains of both mouse groups, but only the 9-10-week-old mice were able to subdue viral DNA replication and gene expression after 5 days post-infection. Pro-inflammatory cytokine mRNAs of tumor necrosis factor-α, interleukin 1β, and interleukin 6 were highly induced in the brains of the 4-5-week-old mice, suggesting their possible contributions as neurotoxic factors in the age-dependent control of MHV-68 replication of the CNS.

INTRODUCTION

Murine gammaherpesvirus (MHV-68 or γHV-68) is genetically related and biologically similar to the human gammaherpesviruses, Epstein-Barr virus (EBV), and Kaposi's sarcoma-associated herpesvirus (KSHV), which are commonly associated with human cancers (Simas and Efstathiou, 1998). Like other herpesviruses, gammaherpesviruses establish life-long, persistent infection in the host through two distinct life cycle phases: lytic replication and latent infection. Productive lytic infection is characterized by high levels of viral gene expression, viral genome replication, infectious virion production, and host cell lysis. During non-productive latent infection, a limited number of viral genes are expressed, viral genomes are maintained rather than amplified, no infectious virions are produced, and no infected cells undergo cell death. Although latent infection of gammaherpesvirus infection is thought to critically affect life-long infection in the host, lytic replication from periodic reactivation also is important in propagating the virus and replenishing the virus pool within the host organism, ultimately contributing to viral persistence.

Due to the availability of *in vivo* infection and genetic manipulation systems, MHV-68 provides a tractable animal model in which to study gammaherpesvirus pathogenesis and replication *in vivo*. Following intranasal inoculation of MHV-68, transient lytic replication occurs in the lung and is followed by spreads into B cells of the spleen. Characteristic splenomegaly coincides with an increase in the latent infection of spleen B cells 2 weeks post-infection, which resolves at approximately 3 weeks. Thereafter, long-term latency of MHV-68 is found primarily in infected B cells (Sunil-Chandra et al., 1992a; 1992b). Other tissues, such as lung epithelial cells (Stewart et al., 1998) and peritoneal macrophages (Weck et al., 1999), also can serve as latent reservoirs. Long-term carriers of MHV-68 occasionally develop B-cell lymphoma, similar to human gammaherpesviruses (Sunil-Chandra et al., 1994).

Although gammaherpesviruses are predominantly, but not exclusively, lymphotropic, viral DNAs of the human gammaherpesviruses, EBV, and KSHV also were detected in brain samples from biopsies and autopsies of both healthy and immunodeficient patients (Chan et al., 1999; 2000; Karatas et al., 2008; Weinberg et al., 2005). KSHV was shown to cause encephalitis in immunosuppressed patients (Said et al., 1997). These findings suggest the possible neurotropism and/or neuroinvasiveness of gammaherpesviruses. In fact, the involvement of the nervous system in infectious mononucleosis, an EBV-associated acute infection, is common, and neurological complica-

[1]Division of Biotechnology, College of Life Sciences and Biotechnology, Korea University, Seoul 136-713, Korea, [2]Department of Microbiology, College of Medicine, Hallym University, Chuncheon 200-702, Korea, [3]Department of Anatomy and Neurobiology, College of Medicine, Hallym University, Chuncheon 200-702, Korea, [4]Ilsong Institute of Life Science, Hallym University, Anyang 431-060, Korea, [5]These authors contributed equally to this work.
*Correspondence: moonsong@korea.ac.kr

Received October 30, 2008; accepted November 11, 2008; published online February 5, 2009

Keywords: age-dependency, gammaherpesvirus, multiple sclerosis, neurological diseases, proinflammatory cytokines

tions are cited as the leading cause of death that results from infectious mononucleosis (Hoover et al., 2004). EBV infections of the CNS are linked to various neurological diseases, such as aseptic meningitis, encephalitis, Guillain-Barré syndrome, and multiple sclerosis (MS) (Serafini et al., 2007). In addition, CNS lymphomas in immunocompromised patients with human immunodeficiency virus (HIV) infection are associated strongly with EBV infection. Treatment of HIV-related primary CNS lymphoma (PCNSL) with ganciclovir significantly lowered EBV viral DNA load in the cerebrospinal fluid, suggesting that EBV replicates in patients with PCNSL (Bossolasco et al., 2006). Although accumulating evidence suggests ongoing lytic replication of EBV in the brain (Bossolasco et al., 2006; Weinberg et al., 2005), it remains to be determined whether other cell types in the brain tissue, with the exception of infiltrating B cells, may also harbor lytic or latent virus.

Consistent with the human gammaherpesviruses, MHV-68 originally was isolated from the tissues of a bank vole, *Clethrionomys glareolus*, by passage through neonatal mouse brain (Blaskovic et al., 1980). The virus was subsequently reisolated from the trigeminal ganglia of naturally and experimentally infected mice (Blaskovic et al., 1984; Rajcani et al., 1985). However, upon intracerebral injection of MHV-68 in mice younger than 4 weeks of age, infected mice died by day 7 (Terry et al., 2000). Such high mortality in CNS-infected mice has hampered studies on host responses to MHV-68 and its potential life-long persistence in the CNS. In this study, we examined both the ability of MHV-68 to infect various CNS cell types *in vitro* and age-dependent pathogenesis upon intracerebroventricular (i.c.v.) infection of MHV-68 using two recombinant viruses that expressed either enhanced green fluorescence protein (EGFP) or β-galactosidase (LacZ) as a reporter.

MATERIALS AND METHODS

Cells, viruses, and plaque assays

BHK21 (baby hamster kidney fibroblast cell line), Vero (green monkey kidney cell line), Neuro2A (murine neuroblastoma cell line; ATCC, CCL-131), C6 (rat astrocyte glioma cell line; ATCC, CCL-107), and BV-2 (murine microglial cell line; provided by Dr. Won-Ki Kim, Korea University) cells were propagated in complete Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (HyClone) and penicillin/streptomycin (10 units/ml). MHV-68 virus was obtained originally from the American Type Culture Collection (VR1465). Working virus stocks were grown by infecting BHK-21 cells at a multiplicity of infection (MOI) of 0.05. MHV-68/LacZ and MHV-68/EGFP, recombinant MHV-68 viruses expressing LacZ and EGFP, respectively, were kindly provided by Dr. Ren Sun (University of California at Los Angeles, USA). Viral titers were determined by plaque assay using Vero monolayer cells overlaid with growth medium containing 1% methylcellulose as previously described (Lee et al., 2007a).

Mice, intracerebroventricular injection, and survival analysis

BALB/c mice were obtained from either Orient Bio (Korea) or the Experimental Animal Center, Hallym University (Korea). The animals were fed a commercial diet and water *ad libitum* under controlled temperature, humidity, and lighting conditions. Procedures involving animals and their care were conducted in accord with institutional guidelines that comply with international laws and policies (NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No.85-23, 1996). Two groups of mice between 4-5 weeks and 9-10 weeks of age were i.c.v. injected in the left hemisphere of the brain with 11 μl of either 550 PFU MHV-68/LacZ virus or serum-free DMEM media. This procedure was performed using a Hamilton syringe following anesthesia with an intraperitoneal injection of 2,2,2-tribromoethanol in 2-methyl-2-butanol (Sigma-Aldrich, USA; 250 mg/kg). To calculate the age-dependent survival rates of i.c.v.-infected mice (n = 41 or 42) and mock-infected mice (n = 7 or 9), Kaplan-Meier survival statistical analyses were performed using MedCalc for Windows, version 9.5.0.0 (MedCalc Software, Belgium).

Tissue processing, LacZ staining, and immunohistochemistry

Brain tissues were processed for immunohistochemistry as described previously (Kwak et al., 2005). Briefly, mock- or MHV-68/LacZ virus-infected animals were perfused via the ascending aorta with 200 ml of 4% paraformaldehyde in phosphate buffer (PB). The brains were removed, post-fixed in the same fixative for 4 h, and rinsed in PB containing 30% sucrose at 48°C for 2 d. Thereafter, the tissues were frozen and sectioned with a cryostat, and consecutive 30-μm sections were collected into six-well plates containing phosphate buffered saline (PBS). Free-floating sections were first incubated with 10% normal horse serum for 30 min at room temperature and then incubated with LacZ staining solution [5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6{\cdot}3H_2O$, 2mM $MgCl_2$, 0.01% Na deoxycholate, 0.02% NP-40, and 1 mg/ml X-gal] at 37°C for 4 h to overnight Following three washes, sections were incubated with rabbit anti-M9 (viral small capsid protein, 1:200), rabbit anti-glial fibrillary acidic protein (GFAP, 1:100, Lab vision), or goat anti-vimentin antibody (1:100, chemicon) in PBS containing 0.3% Triton X-100 and 2% normal goat serum overnight at room temperature. After washing three times for 10 min with PBS, sections were incubated sequentially, in goat anti-rabbit or rabbit anti-goat IgG and ABC complex (Vector Laboratories, USA), diluted 1:200 in the same solution as the primary antiserum. Between the incubations, the tissues were washed with PBS three times for 10 min each. The sections were visualized with 3,3′-diaminobenzidine (DAB) in 0.1 M Tris buffer and mounted on gelatin-coated slides. The immunoreactions were observed under the Axioscope microscope (Carl Zeiss, Germany). To establish the specificity of the immunostaining, a negative control test was carried out with preimmune serum instead of primary antibody. The negative control resulted in the absence of immunoreactivity in any structures.

Viral RNA isolation and RT-PCR analysis

Total RNAs were extracted from tissues using TRI reagents (Molecular Research Center, USA) according to the manufacturer's instructions. The cDNAs were synthesized using SuperScript III RNaseH reverse transcriptase (Invitrogen) as described elsewhere (Kwon et al., 2003). The synthesized cDNAs were subjected to RT-PCR analysis using a series of cytokine-specific primers as follows (Kwon et al., 2003; Lee et al., 2007b): mTNF-α (F; 5′-TCTCATCAGTTCTATGGCCC-3′, R: 5′-GGGAGTAGACAAGGTACAAC-3′), mIL-1β (F: 5′-TTGACGGACCCCAAAAGATG-3′, R: 5′-AGAAGGTGCTCATGTCCTCA-3′), mIL-6 (F: 5′-GTTCTCTGGGAAATCGTGGA-3′, R: 5′-TGTACTCCAGGTAGCTATGG-3′), and mIFN-β (F: TTACACTGCCTTTGCCATCC; R: ACTGTCTGCTGGTGGAGTTCAT) and viral transcript-specific primers for RTA and ORF57 (Lee et al., 2007a).

Viral DNA isolation and analysis by quantitative real-time PCR

Genomic DNAs, including viral DNAs from cells or tissues,

were isolated by a standard method of phenol:chloroform extraction and ethanol precipitation of the harvested cells (Song et al., 2005). Real-time PCR of genomic DNA (50 ng) was performed in triplicate on an iCycler (Bio-Rad) as a 20-μl reaction mixture using SYBR green and specific primers for ORF 56-specific primers (nt 75,598-75,783) (Lee et al., 2007). Real-time PCR was run at 50°C for 2 min and 45 cycles at 95°C for 10 s, 58°C for 15 s, and 72°C for 20 s, followed by melting curve analysis. The results were analyzed according to the manufacturer's instructions.

Antibodies and western blotting

Cell extracts were examined using the following primary antibodies: rabbit polyclonal antibody to ORF26 (1:500), ORF45 (1:400), or M9 (1:500) and mouse monoclonal antibody to α-tubulin (1: 3000). Anti-rabbit or anti-mouse IgG conjugated with horseradish peroxidase (Santa Cruz Biotechnology) was used as a secondary antibody. The ECL Plus System chemiluminescence detection method (Amersham Pharmacia Biotech) was used for western analysis.

RESULTS

Neuronal cells, microglial cells, and astrocytes support lytic MHV-68 *in vitro*

Infection with recombinant virus harboring a reporter gene expression cassette in the viral genome is widely used to monitor viral infection both *in vitro* and *in vivo*. In this study, recombinant MHV-68 viruses expressing either the EGFP gene (MHV-68/EGFP) or the LacZ gene (MHV-68/LacZ) were used to monitor viral infection *in vitro* and *in vivo*, respectively (Fig. 1A). Each reporter cassette was inserted at the left end of the viral genome (nt 1,891) where the insertion has little influence on either virus growth or neighboring gene expressions (Song et al., 2004).

Gammaherpesvirus infections usually result in two distinct outcomes: lytic replication or latency. To examine the infection patterns of MHV-68 in the cells found in the CNS, Neuro2A (murine neuroblastoma cells), BV-2 (murine microglial cells), and C6 (rat glioma astrocytes) were infected with increasing doses of MHV-68/EGFP at MOIs equivalent to 0.01, 0.1, 0.2, and 1.0 (PFU/cell). BHK21 cells, fully permissive to MHV-68, were used as a positive control. At 3 days post-infection, infected cells were analyzed for EGFP and viral gene expression, as well as viral DNA replication, and the culture supernatants were analyzed for infectious virion production (Figs. 1B-1E). Both neuronal and microglial cells supported limited viral gene expression, viral DNA replication, and virion production, more prominently at low MOIs than at high MOIs. In contrast, astrocytes were highly permissive to MHV-68 lytic infection at all MOIs, showing severe cytopathic effects (CPEs) in infected cells and producing the highest virus titers among all cell lines tested (Fig. 1E). Notably, infected Neuro2A cells did not show any apparent signs of CPEs during the course of infection, despite strong EGFP expression at higher MOIs. This result suggests a unique pattern of viral infection in neuronal cells. Taken together, our results demonstrate that neuronal cells, microglial cells, and astrocytes support lytic infection of MHV-68 to various extents.

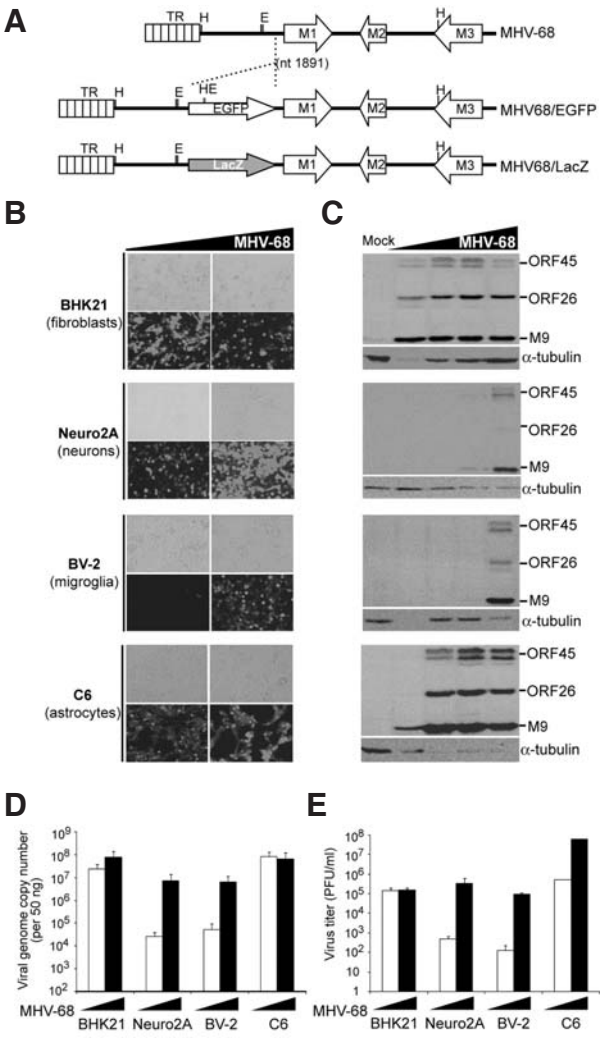

Fig. 1. Susceptibility of neuronal cells, microglial cells, and astrocytes to MHV-68 infection *in vitro*. (A) Schematic diagram of the genomic structures of the MHV-68/EGFP and MHV-68/LacZ viruses. Shown is the 5′ end of the viral genome encompassing terminal repeats and ORFs M1-3. The reporter gene expression cassette driven by the CMV promoter was inserted at nt 1,891. Neuro2A, BV-2, C6, and BHK21 cells were infected with increasing doses of MHV-68/EGFP and harvested on day 3 for EGFP expression (B), viral gene expression (C), viral DNA genome replication (D), and infectious virion production (E). (B) Infected cells were monitored for EGFP expression by fluorescence microscopy. Shown are representative images from cells infected with MHV-68 (MOIs = 0.1 and 1). At higher MOI (PFU/cell), lower levels of GFP were detected in BHK21 and C6 cells due to severe CPEs. (C) Viral gene expression in infected cells (MOIs = 0.01, 0.1, 0.2, and 1) were analyzed by immunoblotting using anti-ORF45 (viral tegument protein), anti-ORF26 (capsid protein), and anti-M9 (small capsid protein) antibodies. α-tubulin was used as a loading control. (D) Viral DNA replication in the infected cells was analyzed by real-time PCR. (E) The cultured supernatants from infected cells were assayed for infectious virion production by plaque assay.

Age-dependent mortality and virus replication of MHV-68 in i.c.v.-injected mice

Terry et al. (2000) showed that intracerebrally-infected mice younger than 4 weeks of age became immediately moribund and died between days 5-7 post-infection by MHV-68. To examine the effects of age-dependent viral replication in the CNS, we performed i.c.v. injection of MHV-68/LacZ (550 PFU) into two groups of mice, 4-5 weeks (n = 42) and 9-10 weeks (n = 41)

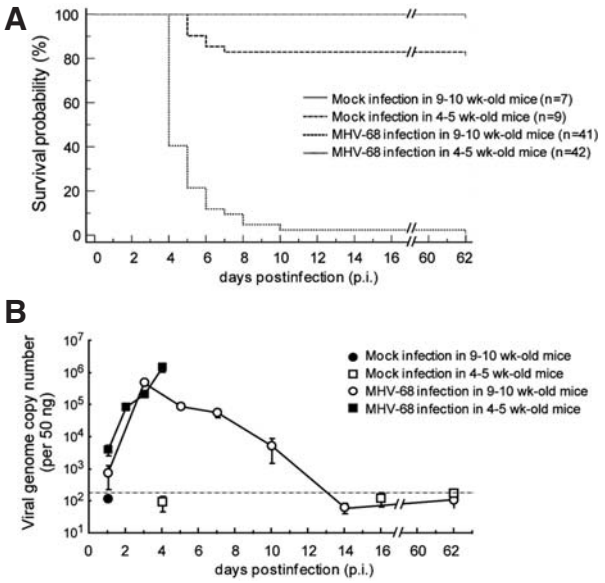

Fig. 2. Age-dependent mortality and viral replication of MHV-68/LacZ in the CNS. (A) Survival analysis of 4-5-week-old and 9-10-week-old mice that were intracerebroventricularly (i.c.v.) injected with either mock- or MHV-68/LacZ. Upon i.c.v. injection of MHV-68/LacZ (550 PFU), survival of the infected mice was monitored daily and Kaplan-Meier survival analysis was performed. (B) Mice infected with MHV-68/LacZ were sacrificed on the days indicated and genomic DNA from the brain tissues was extracted for real-time PCR to measure viral genome copy number. Shown are representative results from real-time PCR experiments in triplicate. The dotted line indicates the detection limit.

of age, and then monitored their survival rates daily until 62 days post-infection (Fig. 2A). An age-matched group of mice (n = 9 or 7) was i.c.v. injected with serum-free media as a negative control. Consistent with the previous study, all of the 4-5-week-old infected mice immediately became unwell: approximately 60% of these mice died within 5 days and more than 95% were dead within 7-10 days. Only one mouse survived until day 62 and did not show any signs of infection. By days 3-5, the 9-10-week-old-infected mice showed clinical signs of infection, including ruffled fur, reduced activity, and weight loss, but recovered after 10-14 days, resulting in higher survival rates (> 80%). Similar results for the age-dependent survival analysis using both MHV-68/LacZ and wild-type MHV-68 were obtained in more than three independent experiments (n = 12-30) (data not shown). These results suggest that age influences viral replication directly or via an age-dependent decline or increase in the immune responses of the CNS upon i.c.v. injection of MHV-68.

To measure the extent of viral replication in infected mice, both mouse groups (n = 4-7) were infected with MHV-68 (550 PFU) or serum-free media and sacrificed on the days indicated (Fig. 2B). Total genomic DNA was isolated from the brain tissues and subjected to real-time PCR using specific primers with serially diluted standards. Viral DNA replication in 4-5-week-old mice continued to increase until day 4; after day 4, it was unreliable to collect brain samples from these mice due to their high mortality rate. However, in the 9-10-week-old mice, viral genome replication reached a peak around 3-5 days, gradually decreased after day 5, and was undetectable by days 10-14 (Fig. 2B). The levels and kinetics of viral replication in the brains of these infected mice were comparable for the first 3-4 days, suggesting little difference between the two age groups in supporting viral lytic replication. Similar levels of viral DNA were also detected in the right side of the infected hemisphere, indicating an even spread of infection regardless of the site of injection (data not shown). Therefore, these results suggest that an age-dependent decline or increase in host responses controls viral replication, producing dramatically different MHV-68 infection outcomes in the two mouse groups.

Age-dependent viral gene expression of MHV-68 in the CNS

MHV-68/LacZ expresses β-galactosidase as a reporter that renders infected cells blue following incubation with X-gal. The expression of β-galactosidase after MHV-68/LacZ infection was shown to be dose-dependent, providing an excellent tool to quantitatively and qualitatively monitor viral replication both *in vitro* and *in vivo* (unpublished data). To examine the progression of viral infection in the brain upon i.c.v. injection of MHV-68/LacZ (550 PFU) of the two mouse groups, we measured β-galactosidase gene expression and viral gene expression in infected brain tissues (Fig. 3). In 4-5-week-old mice (n = 2-3), immunoreactivity against M9 (viral small capsid protein) was first detected on day 2 in ependymal cells and the alveolus (the nerve fiber bundle of hippocampal neurons forming the fornix) (Fig. 3A). The signals from both M9 and LacZ were also spread to the hippocampal regions via the alveolus on days 3 and 4. The LacZ signal was not colocalized with GFAP or vimentin immunoreactivity, known as specific markers for glial cells and astrocytes, suggesting that neuronal cells, rather than microglia or astrocytes, may be the major location of MHV-68 infection in 4-5-week-old mice (Fig. 3B) as well as in 9-10-week-old mice (data not shown). In 9-10-week-old mice, similar patterns of viral infection were observed, although the spread of the virus was generally more restricted to regions proximal to the injection site (Figs. 3C and 3D). Consistent with the results of viral DNA replication, M9 viral gene and LacZ expression levels were decreased by day 10 (Fig. 3C) and were undetectable by day 14 (data not shown), suggesting successful control of viral gene expression by 2 weeks post-infection. However, the peak of viral gene expression was delayed to 5-7 days compared with viral DNA replication. M9 immunoreactive structures were detected in perivascular regions near the ventricles; viral gene expression was occasionally detected in the striatal neurons, a distant locus from the site of injection (Fig. 3D).

Age-dependent pro-inflammatory cytokine responses to MHV-68 infection in the brain

As shown by real-time PCR and immunohistochemistry analyses, the levels of MHV-68/LacZ lytic replication were comparable in both mouse groups for 3-5 d. However, only the 9-10-week-old mice were able to control and resolve viral replication thereafter whereas the 4-5-week-old mice displayed a high mortality rate (> 90%) after 5 days, suggesting different host responses to viral brain infection. To examine the possible contribution of pro-inflammatory cytokines in viral pathogenesis in the brain, total RNAs isolated from brain tissues were subjected to either real-time RT-Q-PCR or RT-PCR for interferon-β (IFN-β), tumor necrosis factor-α (TNF-α), interleukin 1β (IL-1β), and interleukin 6 (IL-6) as well as for viral lytic mRNAs (i.e., RTA, a lytic switch gene and ORF57, an early gene) (Fig. 4). The viral lytic transcripts of RTA and ORF57 expressed and increased over time in both 4-5-week-old and 9-10-week-old mice (Fig. 4A). The induction folds of RTA expression were higher in 9-10-

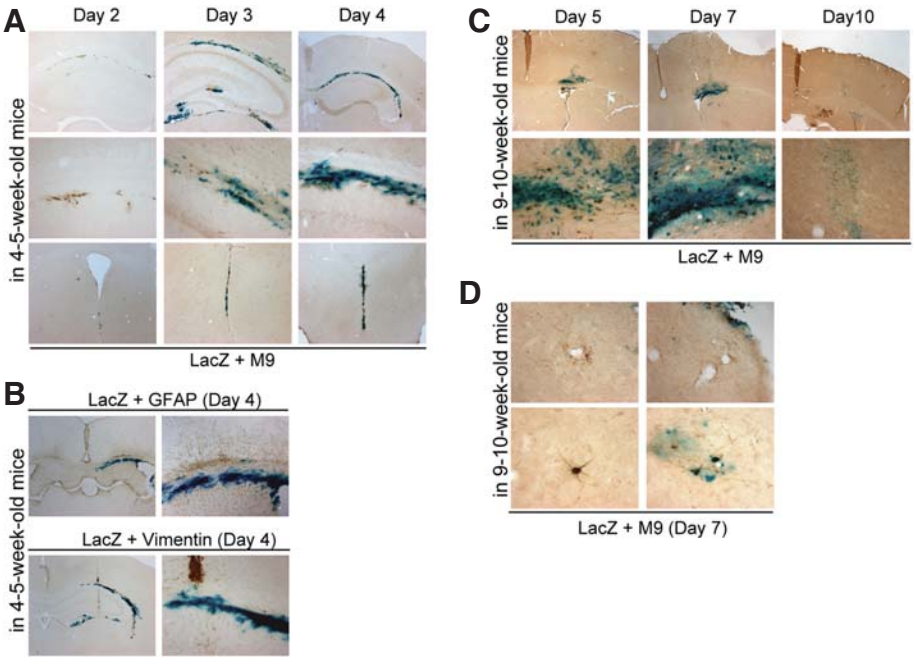

Fig. 3. Viral gene and LacZ expression in the CNS of mice infected with MHV-68/LacZ. (A) MHV-68/LacZ infection in the CNS of 4-5-week-old mice. Histochemical staining for β-galactosidase and M9 expression is shown in brain tissues of infected 4-5-week-old mice on day 2, day 3, and day 4. Magnification ×25 (c, f, g, i), ×50 (a, d), ×100 (b) and ×200 (e, h). (B) Histochemical staining for β-galactosidase and GFAP or vimentin expression in brain tissues of infected 4-5-week-old mice on day 4. The β-galactosidase-positive cells are shown in blue and do not overlap with either GFAP or vimentin (brown). Magnification ×25 (a, c) and ×100 (b, d). (C) MHV-68/LacZ infection in the CNS of 9-10-week-old mice. Histochemical staining of M9 + β-galactosidase expression on day 5, day 7, and day 10 in 9-10-week-old mice. Magnification ×25 (a, c, e) and ×200 (b, d, f). (D) Perivascular expression of M9 near the ventricles (a and b) and M9 and β-galactosidase expression levels in striatal neurons (c and d) of the brain tissues of infected 9-10-week-old mice on day 7. Magnification ×200 (a, b) and ×400 (c, d).

week-old mice than in 4-5-week-old mice, while ORF57 expressions were comparable in the two mouse groups. The IFN-β transcript was not expressed in either group compared with age-matched, mock-infected mice; however, it was significantly induced in sendai virus (SeV)-infected NIH3T3 cells, which were used as a positive control (Fig. 4B). Samples from 4-5-week-old mice showed higher expression levels of TNF-α and IL-1β by day 3 than from 9-10-week-old mice (Fig. 4C). Interestingly, IL-6 expression was constitutively expressed in mock-infected 9-10-week-old mice and decreased over time by MHV-68/LacZ infection (Fig. 4C), while IL-6 was highly induced by day 3 in MHV-68/LacZ-infected 4-5-week-old mice. These results suggest that differentially expressed pro-inflammatory cytokines, TNF-α, IL-1β, and IL-6 in two age groups, may contribute to age-dependent viral pathogenesis upon MHV-68 infection, via playing an either neuroprotective or neurotoxic role.

DISCUSSION

In this study, we examined the susceptibility of neuronal cells, microglial cells, and astrocytes to MHV-68 infection *in vitro*. We also assessed age-dependent viral pathogenesis following i.c.v. injection of MHV-68 in 4-5-week-old and 9-10-week old mice. Our results showed that both neuronal (Neuro2A) and microglial (BV-2) cells were permissive to MHV-68 infection to limited extents, while astrocytes (C6) efficiently supported lytic replication to levels comparable to those in fully permissive fibroblasts. Direct injection of MHV-68 into the CNS resulted in a high mortality rate in the 4-5-week-old mice and in prolonged survival in the 9-10-week-old mice. TNF-α, IL-1β, and IL-6 mRNAs were up-regulated in the brains of the 4-5-week-old mice, compared to those in the 9-10-week-old mice. These results suggest that highly induced TNF-α, IL-1β, and IL-6 may function as neurotoxic factors in young mice, while decreased IL-6 may provide neuroprotective effects in adult mice.

Neurotropism and age-dependent neuropathogenesis of MHV-68: a model system in which to study the life-long persistent infection of gammaherpesvirus in the CNS

Although gammaherpesviruses are known primarily to be lymphotropic, they have been shown to infect diverse tissue/cell types, including epithelial cells, endothelial cells, monocytes, macrophages, dendritic cells, microgial cells, and astrocytes. These cells harbor either lytic or latent viruses and serve as dynamic reservoirs of virus infection *in vivo*. As EBV viral DNA is found in brain samples from MS patients (Serafini et al., 2007) and KSHV viral DNA can be found in both dorsal root ganglia of Karposi's sarcoma patients as well as normal brain samples (Corbellino et al., 1996), the potential for gammaherpesviruses to interact with the CNS was sought in this study. Our *in vitro* infection data demonstrate that MHV-68 successfully entered and replicated to a limited extent in cultured neuronal cells and this is the first to report gammaherpesvirus replicating in neuronal cells. The infection pattern was unique in that MHV-68 infection did not cause typical CPEs and was able to persist for a longer period. The similar infection patterns were also observed in MHV-68 infection of cultured human neuronal cells (unpublished data, H.J. Cho and M.J. Song). We are currently characterizing the nature of MHV-68 infection in neuronal cells.

Although intranasal infection of MHV-68 is not normally neuroinvasive due to the blood-brain barrier (BBB), MHV-68 was both present and able to replicate in the brains of IFN-α/-β-$R^{0/0}$ mice (Terry et al., 2000), suggesting that MHV-68 may gain access to the CNS from the periphery under certain immunodeficient conditions. However, in the previous study, the authors were not able to study the long-term effects of viral infection in the CNS because only a small number of mice up to 4 weeks of age were tested and all died of meningitis shortly thereafter. Our results showed that mice older than 9 weeks of age survived by controlling viral replication at low levels in the brain after two weeks p.i. Less induced TNF-α and IL-1β mRNAs as

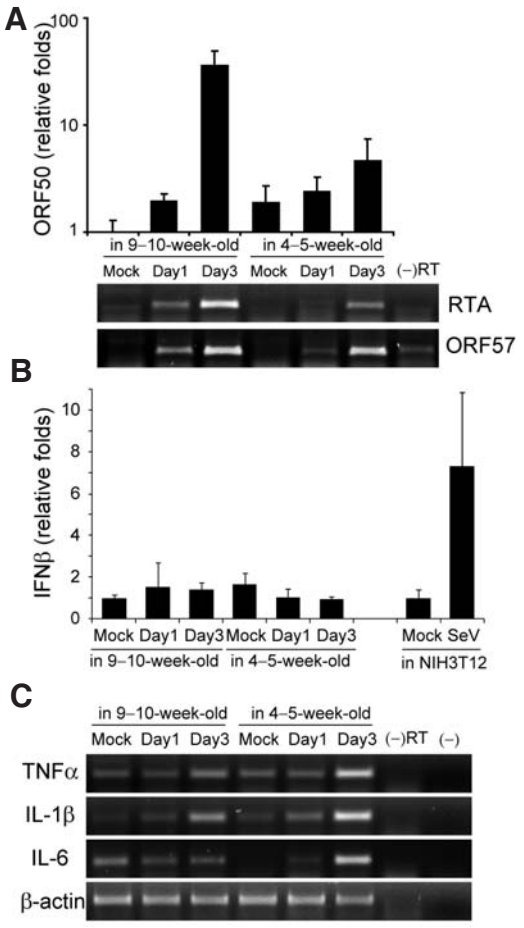

Fig. 4. Age-dependent expression of pro-inflammatory cytokines in MHV-68/LacZ-infected mice. (A) Viral lytic gene expressions in MHV-68/LacZ-infected mice with the ages of 4-5-weeks and 9-10-weeks. Quantitative real-time RT-PCRs using ORF50 (a main coding region of RTA, an immediate early gene)-specific primers in mock- and MHV-68/LacZ-infected mice was conducted; relative ORF50 expression levels (*n*-fold) were calculated based on those of mock-infected 9-10-week-old mice, after being normalized for β-actin levels. Results are presented as the mean of triplicates with their standard deviation. Another set of primers specific for the spliced transcript of RTA was utilized for RT-PCRs and a representative gel picture is shown. The levels of ORF57 (an early lytic gene) transcripts were also measured via RT-PCR, and shown was a representative picture. (B) Real-time RT-Q-PCR of IFN-β mRNA expression in mock- and MHV-68/LacZ-infected mice using β-actin as an internal control. Mock- and sendai virus (SeV, MOI = 4 for 16-h)-infected NIH3T12 cells were used as a positive control. (C) RT-PCR of TNF-α, IL-1β, and IL-6 mRNA expression. β-actin serves as a loading control.

well as lower levels of IL-6 mRNA were detected in 9-10-week-old mice than in 4-5-week-old mice. Considering the nature of life-long gammaherpesvirus infection, these results will provide a plausible model system in which to study the long-term effects of gammaherpesvirus in the CNS. Whether MHV-68 establishes persistent infection in the CNS or alternatively spreads elsewhere in the body is an open question and remains to be elucidated. Real-time kinetic studies of viral infection using bioluminescence imaging may be excellent approaches to address such an issue. We are currently investigating this hypothesis using another recombinant MHV-68 containing the luciferase expression cassette. Collectively, given the facts that cultured neuronal cells supported lytic replication of MHV-68 and that i.c.v. injection of MHV-68 led to region-specific viral gene expression in the brain, especially in hippocampus, it is highly likely that MHV-68 may have neurotropism *in vitro* and *in vivo*.

Pro-inflammatory cytokine responses: neuroprotective or neurotoxic?

The involvement of viral infections in chronic inflammatory diseases in the CNS has recently been the subject of much interest following reports of EBV infection as a risk factor for both MS and HIV-associated dementia. EBV association in MS is attractive, given that latent infections established by herpesviruses periodically reactivate in a manner similar to the relapsing-remitting symptoms of MS. EBV-specific antibodies have been significantly correlated with cases of MS (Ascherio and Munch, 2000; Ascherio et al., 2001). However, the identity of the CNS cell populations as targets for EBV infection and the nature of the cellular responses evoked by such infections within the CNS are poorly understood. MHV-68 has been reported to infect cultured microglia and astrocytes and induce TNF-α and IL-6 (Taylor et al., 2003). Consistent with this, our *in vitro* data showed that microglia and astrocytes can harbor lytic MHV-68. However, reporter gene expression in ependymal cells, alveolus and a few striatal neurons, not in glial cells, suggests that *in vivo* infection of MHV-68 (550 PFU) into microglia and astrocytes may not be as efficient as it is *in vitro.* Terry et al. (2000) reported that hippocampal pyramidal neurons are predominantly infected following striatal implantation of infected glial cells, although direct intracerebral inoculation of MHV-68 (2×10^4 PFU) infected hippocampal pyramidal neurons, oligodendrocytes, Bergmann glia cells in the cerebellar cortex and neural progenitor cells in the rostral migratory stream. Therefore, the viral concentration/infection route may be a crucial factor to determine the infected cell types, since innate immunity may inhibit the efficient infection of microglial or astrocyte populations when infected with a low concentration of virus *in vivo*.

Microglial cells have been shown to be susceptible to other herpesviruses, including herpes simplex virus (HSV) and cytomegalovirus (CMV), eliciting TNF-α, IL-1β, and/or IL-6 (Lokensgard et al., 2001) as well as to a lymphotropic retrovirus, HIV-1. In the case of HSV infection, both TNF-α and IL-1β appear to provide neuroprotective effects since TNF-α- and IL-1β-knockout mice suffer from higher lethality due to encephalitis with higher viral loads than wild-type mice (Sergerie et al., 2007). In the case of HIV-associated dementia, HIV infection in macrophages and microglia results in neurotoxin secretion [reviewed in (Brabers and Nottet, 2006)]. Two of these neurotoxins are TNF-α and IL-1β that induce neuronal cell death by over-stimulating the NMDA receptor. Therefore, pro-inflammatory cytokines can possess both neuroprotective and neurotoxic functions, which are likely to play important roles in either the development of protective immune responses against viral infection or the progression of damaging inflammation during CNS diseases states. In this study, although elevated levels of TNF-α and IL-1β may account for higher mortality rates in 4-5-week-old mice, infection of MHV-68 in knockout mice lacking such pro-inflammatory cytokines may provide a better understanding of viral replication and pathogenesis. Taken together, our results reveal an age-dependent neuropathogenesis and differential expression of pro-inflammatory cytokines by MHV-68 infection in the CNS and provide a plausible model system

in which to study the long-term effects of viral infection in the CNS and to gain a better understanding of gammaherpesvirus neurotropism and virus-associated neurological diseases.

ACKNOWLEDGMENTS

This work was supported by the Korea Research Foundation Grants funded by the Korean Government (Ministry of Education and Human Resource and Deparment, Basic Research Promotion Fund) (KRF-2007-531-C00044 and R04-2004-000-10048-0 to M.J.S.).

REFERENCES

Ascherio, A., and Munch, M. (2000). Epstein-Barr virus and multiple sclerosis. Epidemiology *11*, 220-224.

Ascherio, A., Munger, K.L., Lennette, E.T., Spiegelman, D., Hernan, M.A., Olek, M.J., Hankinson, S.E., and Hunter, D.J. (2001). Epstein-Barr virus antibodies and risk of multiple sclerosis: a prospective study. JAMA *286*, 3083-3088.

Blaskovic, D., Stancekova, M., Svobodova, J., and Mistrikova, J. (1980). Isolation of five strains of herpesviruses from two species of free living small rodents. Acta Virol. *24*, 468.

Blaskovic, D., Stanekova, D., and Rajcani, J. (1984). Experimental pathogenesis of murine herpesvirus in newborn mice. Acta Virol. *28*, 225-231.

Bossolasco, S., Falk, K.I., Ponzoni, M., Ceserani, N., Crippa, F., Lazzarin, A., Linde, A., and Cinque, P. (2006). Ganciclovir is associated with low or undetectable Epstein-Barr virus DNA load in cerebrospinal fluid of patients with HIV-related primary central nervous system lymphoma. Clin. Infect. Dis. *42*, e21-25.

Brabers, N.A., and Nottet, H.S. (2006). Role of the pro-inflammatory cytokines TNF-alpha and IL-1beta in HIV-associated dementia. Eur. J. Clin. Invest. *36*, 447-458.

Chan, P.K., Ng, H.K., Hui, M., Ip, M., Cheung, J.L., and Cheng, A.F. (1999). Presence of human herpesviruses 6, 7, and 8 DNA sequences in normal brain tissue. J. Med. Virol. *59*, 491-495.

Chan, P.K., Ng, H.K., Cheung, J.L., and Cheng, A.F. (2000). Survey for the presence and distribution of human herpesvirus 8 in healthy brain. J. Clin. Microbiol. *38*, 2772-2773.

Corbellino, M., Poirel, L., Bestetti, G., Pizzuto, M., Aubin, J.T., Capra, M., Bifulco, C., Berti, E., Agut, H., Rizzardini, G., et al. (1996). Restricted tissue distribution of extralesional Kaposi's sarcoma-associated herpesvirus-like DNA sequences in AIDS patients with Kaposi's sarcoma. AIDS Res. Hum. Retroviruses *12*, 651-657.

Hoover, S.E., Ross, J.P., and Cohen, J.I. (2004). Epstein-Barr virus, In Infections of the central nervous system. W.M. Scheld, R.J. Whitley, and C.M. Marra, eds. (Philadelphia: Lippincott Williams & Wilkins), pp. 175-183.

Karatas, H., Gurer, G., Pinar, A., Soylemezoglu, F., Tezel, G.G., Hascelik, G., Akalan, N., Tuncer, S., Ciger, A., and Saygi, S. (2008). Investigation of HSV-1, HSV-2, CMV, HHV-6 and HHV-8 DNA by real-time PCR in surgical resection materials of epilepsy patients with mesial temporal lobe sclerosis. J. Neurol. Sci. *264*, 151-156.

Kwak, S.E., Kim, J.E., Kim, D.S., Jung, J.Y., Won, M.H., Kwon, O.S., Choi, S.Y., and Kang, T.C. (2005). Effects of GABAergic transmissions on the immunoreactivities of calcium binding proteins in the gerbil hippocampus. J. Comp. Neurol. *485*, 153-164.

Kwon, H.J., Lee, K.W., Yu, S.H., Han, J.H., and Kim, D.S. (2003). NF-kappaB-dependent regulation of tumor necrosis factor-alpha gene expression by CpG-oligodeoxynucleotides. Biochem. Biophys. Res. Commun. *311*, 129-138.

Lee, S., Cho, H.J., Park, J.J., Kim, Y.S., Hwang, S., Sun, R., and Song, M.J. (2007a). The ORF49 protein of murine gammaherpesvirus 68 cooperates with RTA in regulating virus replication. J. Virol. *81*, 9870-9877.

Lee, J.Y., Kim, J.Y., Lee, Y.G., Shin, W.C., Chun, T., Rhee, M.H., and Cho, J.Y. (2007b). Hydroquinone, a reactive metabolite of benzene, reduces macrophage-mediated immune responses. Mol. Cells. *23*, 198-206.

Lokensgard, J.R., Hu, S., Sheng, W., vanOijen, M., Cox, D., Cheeran, M.C., and Peterson, P.K. (2001). Robust expression of TNF-alpha, IL-1beta, RANTES, and IP-10 by human microglial cells during nonproductive infection with herpes simplex virus. J. Neurovirol. *7*, 208-219.

Rajcani, J., Blaskovic, D., Svobodova, J., Ciampor, F., Huckova, D., and Stanekova, D. (1985). Pathogenesis of acute and persistent murine herpesvirus infection in mice. Acta Virol. *29*, 51-60.

Said, J.W., Tasaka, T., de Vos, S., and Koeffler, H.P. (1997). Kaposi's sarcoma-associated herpesvirus/human herpesvirus type 8 encephalitis in HIV-positive and -negative individuals. AIDS *11*, 1119-1122.

Serafini, B., Rosicarelli, B., Franciotta, D., Magliozzi, R., Reynolds, R., Cinque, P., Andreoni, L., Trivedi, P., Salvetti, M., Faggioni, A., et al. (2007). Dysregulated Epstein-Barr virus infection in the multiple sclerosis brain. J. Exp. Med. *204*, 2899-2912.

Sergerie, Y., Rivest, S., and Boivin, G. (2007). Tumor necrosis factor-alpha and interleukin-1 beta play a critical role in the resistance against lethal herpes simplex virus encephalitis. J. Infect. Dis. *196*, 853-860.

Simas, J.P., and Efstathiou, S. (1998). Murine gammaherpesvirus 68: a model for the study of gammaherpesvirus pathogenesis. Trends Microbiol *6*, 276-282.

Song, M.J., Hwang, S., Wong, W., Round, J., Martinez-Guzman, D., Turpaz, Y., Liang, J., Wong, B., Johnson, R.C., Carey, M., et al. (2004). The DNA architectural protein HMGB1 facilitates RTA-mediated viral gene expression in gamma-2 herpesviruses. J. Virol. *78*, 12940-12950.

Song, M.J., Hwang, S., Wong, W.H., Wu, T.T., Lee, S., Liao, H.I., and Sun, R. (2005). Identification of viral genes essential for replication of murine gamma-herpesvirus 68 using signature-tagged mutagenesis. Proc. Natl. Acad. Sci. USA *102*, 3805-3810.

Stewart, J.P., Usherwood, E.J., Ross, A., Dyson, H., and Nash, T. (1998). Lung epithelial cells are a major site of murine gammaherpesvirus persistence. J. Exp. Med. *187*, 1941-1951.

Sunil-Chandra, N.P., Efstathiou, S., Arno, J., and Nash, A.A. (1992a). Virological and pathological features of mice infected with murine gamma-herpesvirus 68. J. Gen. Virol. *73*, 2347-2356.

Sunil-Chandra, N.P., Efstathiou, S., and Nash, A.A. (1992b). Murine gammaherpesvirus 68 establishes a latent infection in mouse B lymphocytes *in vivo*. J. Gen. Virol. *73*, 3275-3279.

Sunil-Chandra, N.P., Arno, J., Fazakerley, J., and Nash, A.A. (1994). Lymphoproliferative disease in mice infected with murine gammaherpesvirus 68. Am. J. Pathol. *145*, 818-826.

Taylor, W.R., Rasley, A., Bost, K.L., and Marriott, I. (2003). Murine gammaherpesvirus-68 infects microglia and induces high levels of pro-inflammatory cytokine production. J. Neuroimmunol. *136*, 75-83.

Terry, L.A., Stewart, J.P., Nash, A.A., and Fazakerley, J.K. (2000). Murine gammaherpesvirus-68 infection of and persistence in the central nervous system. J. Gen. Virol. *81*, 2635-2643.

Weck, K.E., Kim, S.S., Virgin, H.I., and Speck, S.H. (1999). Macrophages are the major reservoir of latent murine gammaherpesvirus 68 in peritoneal cells. J. Virol. *73*, 3273-3283.

Weinberg, A., Bloch, K.C., Li, S., Tang, Y.W., Palmer, M., and Tyler, K.L. (2005). Dual infections of the central nervous system with Epstein-Barr virus. J. Infect. Dis. *191*, 234-237.

Wu, T.T., Tong, L., Rickabaugh, T., Speck, S., and Sun, R. (2001). Function of Rta is essential for lytic replication of murine gammaherpesvirus 68. J. Virol. *75*, 9262-9273.